Annexure-3
(27 Pages)

ANNEXURE – VI

(Revised Project Proposal)

PROJECT TITLE

Study of genetic diversity in wild (*Sus scrofa cristatus*) and domestic (*Sus scrofa domestica*) pigs to find level of hybridization between them in the vicinity of Ranthambore National park.

1. **Name & designation of the (Principal Investigator)** : **Dr. N.P.S. Chauhan,** **Investigators**

Dr. S.P. Goyal
(Co-Investigator)

Dr. Parag Nigam
(Co-Investigator)

2. **Address of Investigators** : **Wildlife Institute of India, P.O. Box 18, Chandrabani, Dehradun.**

3. **Advisor** : **Director**
Wildlife Institute of India, P.O. Box 18,
Chandrabani, Dehradun.

4. **Name of the Institution/ organization collaborating in the Project** : **Wildlife Institute of India**
Uttaranchal/Rajasthan Forest Departments

5. **Field locations** : **Ranthambore National Park.**

6. **Dates & Duration of the Project** : **1 Year, 6 months (2009-10)**

7. **WII's Research Development Plan: Thrust Areas** : **5.2 Monitoring of carnivores, prey populations and habitats**

5.5 Strengthening of wildlife forensic and conservation genetics capability

8. **Amount of funding requested:**

Total amount of financial layout:	**Year I**	**Rs. 14,95,000**
	Year II	**Rs. 1,66,000**
	Total:	Rs. 16,61,000

Rounded off to Rs. 16,60,000.00 ***(Rupees Sixteen Lakh, SixtyThousand only)***

CP16

9. **Following documents are enclosed:**

Statement I - Summary

Statement II - Project Proposal
  - **2.1 Introduction**
  - **2.2 Study Area**
  - **2.3 Objectives**
  - **2.4 Justification**
  - **2.5 Methodology**
  - **2.6 References**
  - **2.7 Work schedule**

Statement III - Project budget and justification.

Signature of Investigators

(N.P.S. Chauhan) **(S.P. Goyal)**

(Parag Nigam)

2010

Reviewer's comments on the proposal & PIs response

S.No.	Reviewer	Comments	Justifications/Recommendations
1.	**International – I :** Robert C. Fleischer, Ph.D. Head, Center for Conservation and Evolutionary Genetics National Zoological Park & National Museum of Natural History Science Building National Zoological Park Smithsonian Institution PO BOX 37012 MRC 5503 Washington, DC 20013-7012 email: fleischerr@si.edu	The proposal looks like it will yield some interesting results regarding introgression of domestic pig mtDNA into wild pig populations near Ranthambore National Park.	This proposal will allow us to understand if there is any introgression of domestic pig into wild pig.
		It needs to be established first that mtDNA sequences differ between domestic and wild pigs in the region, and it needs to be kept in mind that mtDNA control region sequences are haploid and maternally inherited, so hybridization of male domestic pigs and female wild pigs in nature will not allow detection of hybridization. For a full assessment of hybridization, nuclear markers, such as microsatellites, should provide resolution of the paternal contribution.	mtDNA and microsatellite markers have been used for understanding introgression(hybridization) in pigs. A feasibility of using mtDNA will be explored as it has been reported in pigs, otherwise microsatellites widely applicable for such studies will be used for this purpose. Accordingly, use of microsatellites has been included in the project (page 10).
		I was hoping for more explicit preliminary results to show capability of amplifying and sequencing the mtDNA control region, and also that the domestic and wild pigs differ. Nonetheless, the proposal is well-written and I believe the work will produce some interesting results and is worthy of funding.	Findings of the project would indicate the capability of using mtDNA for this purpose. **The proposal has been found worth of funding.**
2.	**National – I :** Dr. Sher Ali PhD, FNA, FASC, FNASc, Av-Humboldt Fellow, Germany, Senior Staff Scientist and Chief Molecular Genetics Laboratory National Institute of Immunology New Delhi - 110 067, India email: alisher@nii.res.in	The total cost of the project is for duration of one year. The theme of the work itself is not bad. In fact, if pursued in a real scientific manner, **this project would provide a great deal of valuable information.**	Reviewer has visualized the importance of this study and revised in the light of his comments.
		As you may be aware, the project does not have copy of the CV of PI and therefore, I could not see what all s/he has published thus far. Therefore, It is very difficult to fathom if s/he is really trained enough to undertake the proposed study.	Two of the PIs have knowledge of conservation genetic and trained for using required genetics aspects.
		On the face of it, if this project is funded, nothing worthwhile would come out that I am very sure about. This is because the PI only wishes to sequence the D-loop region of the mitochondrial genome. That would surely not tell us the extent of inter-mixing of two gene pools (domestic and wild ones). Such study needs multi-facet approach and a bit mix of bioinformatics to get the best out of the project.	Along with mtDNA, revised proposal has included other widely used microsatellites for such approaches (page 10) and bioinformatics software **(page10)** for achieving project objectives.
		If about 5-10 known genes having species specific sequence organization are taken into consideration along with several satellite markers and mt genome and then organization and expression studies are undertaken, that would be most appropriate. It may be noted that taking the blood samples, and analysing the chromosomes either directly from the blood or from the culture would ensure as to how many of the animals are actual hybrid having 37 chromosomes	Instead of using chromosome based approach which would provide only if there are any hybrids, Therefore, the present project has included use of microsatellites in revised proposal, as data would provide other insights of population genetics in addition to hybridization.
		In fact the study may go beyond because one would surely want to know how often mating takes place between the F1 (hybrid) and domestic or wild animals. This is surely a good theme of work involving beautiful genetically concepts. However, you need a skilled pilot to fly F-16 and not an ordinary cab driver.	This would be possible to address by using multilocus genotyping and has been included in the proposal.

C010

S.No.	Reviewer	Comments	Justifications/Recommendations
3.	**National – II :** Dr. Ajith Kumar Scientist Centre for Wildlife Studies 1669, 31st Cross, 16th Main Banashankari, 2nd Stage Bangalore – 560 070 email: ajith@ncbs.res.in	As said in the proposal, it is well known that wild and domestic pigs interbreed extensively. **A proper assessment of the nature and extent of interbreeding, and the factors that influence these would be of considerable management and theoretical interest.**	Though it is well known that wild and domestic pigs interbreed but no data on its extent is available. Knowing genetic purity of wild pig is important conservation and management issues. Therefore the present project would provide extent of interbreeding between wild and domestic pigs in dry tropical deciduous habitat
		Much of the introduction is of a general nature, and does not lead to a question that the proposal poses.	Much of the significance of such study is to understand and maintain genetic diversity of wild animals. The importance of the study has been clearly indicated on page 5 and 6. As there is an interaction of wild and domestic pigs in the outer fringes of RNP, there is a need to understand if there is a inter breeding between wild and domestic pigs in addition to document genetic diversity. Accordingly, the objectives have bee framed **(page 8)** instead of questions.
		In fact, there is no clear statement of objectives or questions posed except "to study the genetic diversity of wild and domestic pigs…." and "to find the level of hybridization between wild and domestic pigs based on genetic variability" – both these objectives are of a very general nature. The parameters for assessing genetic variability and interbreeding are not defined, nor is there any mention of the factors that might influence these.	The project is basically aimed to document level of genetic diversity which has conservation and management significance and determine extent of interbreeding if any among wild and domestic pigs around RNP. Parameters required to be used have been indicated in the revised proposal **(page 9-11)**.
		Methods of extraction and analysis of DNA are poorly described (some of it past tense, may be pasted from somewhere else), and references given more than 10 years old!	Indicated methods are widely used in genetic studies based on suggested protocols of the kits and Bio Robot of Qiagen, Germany
		There is no mention of statistical analysis of data.	Incorporated in revised proposal **(page 10)**.
		The most important methods section ends rather abruptly, I assume to beat the deadline.	Accordingly it has been revised.
		There is only casual justification of the budget. How many samples would be collected? What is cost of analysis of one sample? How many km would be traveled?	No. of samples to be collected are indicated at page 9 and estimated cost would be of Rs. 12.2 lakhs which has been indicated in the budget. It is difficult to say how many km would be traveled at this juncture.
		In my opinion, although the issue is important this proposal does not do justice to it.	Proposal has been modified accordingly in view of comments of the reviewers.

CP10

Study of genetic diversity in wild (*Sus scrofa cristatus*) and domestic (*Sus scrofa domestica*) pigs to find level of hybridization between them in the vicinity of Ranthambore National park.

Key words: Wild pigs, domestic pigs, breeding, genetic diversity, hybrids, conservation.

STATEMENT-I

SUMMARY

The Project aims at studying genetic diversity in wild (*Sus scrofa cristatus*) and domestic (*Sus scrofa domestica*) pigs to find level of hybridization between them in the vicinity of Ranthambore National park. From the year 2004 onwards, systematic study has been conducted on the ecology and behaviour of wild pigs in and around the park. It has been observed that wild pigs stray out of Ranthambore National park, raid agricultural crops and utilises the agro-ecosystem in peripheral villages for food resource and shelter. While this study was conducted, it was found that wild pigs were increasingly straying out of park into the forest-village interface areas and coming in contact with domestic pigs and perhaps breeding with them. As a result, there may be genetic hybridization between the wild and domestic pig populations. Domestic pigs normally have 2n = 38 chromosomes and the wild pig has 2n = 36. These domestic pigs have been found developing physical characteristics that resemble with wild pigs. Thus breeding and genetic hybridization between wild and domestic pigs is seriously impacting the wild population. Therefore it is very important to conduct a study on genetic diversity in wild and domestic pigs to find level of hybridization between them in the vicinity of Ranthambore National park. In the present study, we aim to analyze the feasibility for detection of hybridized pigs using D loop region of mtDNA gene following the study of Grossi *et al.* (2006) and use multi-locus genotype data along with bioinformatics software to understand population genetics as well as hybridization in pigs. The project study on genetic diversity in wild and domestic pigs will provide a basis for long-term conservation of wild pig populations.

CP10

STATEMENT-II

2.1 INTRODUCTION

The wild pig (*Sus scrofa scrofa*) is one of the most widely distributed large mammals. It has always been associated itself with man, and successfully utilises the human altered landscape (Fadeev, 1981 and Erkinaro *et al.*, 1982). Being a resilient and fast breeding animal, it is capable of expanding and establishing its population in new areas (Erkinaro *et al.*, 1982 and Ahmed, 1991). The species is also known for its biological and ecological plasticity (Tisdell, 1982 and Ramdas, 1987). From the year 2004 onwards, systematic study has been conducted on the ecology and behaviour of wild pigs in man-altered protected areas-village interface situations. Under the ongoing project, study on spatial and temporal distribution, diurnal activity, population status, socio-biology, habitat use, ranging patterns, seasonal changes in the dietary intake and feeding habits, nature and extent of agricultural crop damage, socio-economic aspects, reproductive biology and health aspects of wild pigs has been conducted. A wild pig-proof power fence and mass capture techniques for this species have also been developed.

Interestingly, it has been observed that wild pigs stray out of Ranthambore National park, raid agricultural crops and utilises the agro-ecosystem in peripheral villages for food resource and shelter. They have been increasingly coming in contact with domestic pigs (*Sus scrofa domestica*) in the forest-village interface areas and perhaps breeding with domestic pigs. As a result, there may be genetic hybridization among the wild and domestic pig populations. Domestic pigs normally have 2n = 38 chromosomes and the wild pig has 2n = 36 (Bosma, 1976). Despite these differences in chromosome number, these animals can mate and produce fertile hybrids. The hybrid has 37 chromosomes, and it is morphologically similar to the wild pig (Lui, 2000). Hybrids can successfully breed with the wild and domestic pigs or with other hybrids, generating offspring with 36, 37 or 38 chromosomes. Researchers have been studying and analyzing the genetic intraspecific and species-specific diversity of animals of economic interest and of wild animals. For studies of genetic differentiation, mitochondrial DNA (mtDNA) has been widely used as a tool to characterize races and determine genetic relationships (Brown *et al.*, 1989; Pegoraro *et al.*, 1996; Watanobe *et al.*, 1999). So it is very important to know the level of inbreeding between the wild and domestic pig populations in and around Ranthambore National park for conservation of this endangered species.

There is lot of emphasis to witness such phenomenon of genetic diversity and variability among variety of species. Increasing artificial alteration of landscape and intensive wildlife management may serve as potential forces for genetic change within populations of these species. Several

C810

Chromosome numbers are known to vary among wild, domestic and hybrids pigs, therefore its applicability has been reported for use in differentiating these animals (Bosma, 1976; Lui, 2000). The mtDNA is maternally inherited and has widely been applied for studying population genetics of different populations, phylogeny and phylo-geography of species (Avise, 1994). Since mtDNA is maternally inherited, therefore its use in determination of hybridization has very limited applicability. In a recent study on pigs, nucleotide differentiation in D loop region in the mtDNA has been reported among wild, domestic and hybridized animals (Grossi *et al.*, 2006) and they suggested that it is possible to use these differences for pig differentiation. Another genetic tool most widely used in population differentiation as well as in hybridization is the use of microsatellites or short tandem repast (STR) which have been proved to be polymorphic markers (Pierpaoli *et al.*, 2003). Therefore, in the present study, we aimed to analyze the feasibility for detection of hybridized pigs using D loop region of mtDNA gene as reported by Grossi *et al.* (2006) and use multi-locus genotype data along with bioinformatics software to understand population genetics as well as hybridization in pigs in and around Ranthambhore National Park.

To get a first insight into the genetic structure and diversity of these straying wild pigs, investigations on domestic and wild populations are essentially required. Therefore we need to conduct a study on genetic diversity in wild (*Sus scrofa cristatus*) and domestic (*Sus scrofa domestica*) pigs to find level of hybridization between them in the vicinity of Ranthambore National park is necessary. The project study on genetic diversity of wild and domestic pigs will provide a basis for long-term conservation of wild pig populations. The results of this work will provide the database on genetics of wild and domestic pigs for use by wildlife managers and scientists dealing with such problems in different states.

2.2 STUDY AREA

The study will be conducted in and around the Ranthambore National park, which is situated in the Sawai Madhopur district of eastern Rajasthan. The total area of the Reserve is 1334.6 sq. km. and falls into the park area and its buffer, revenue land, reserve forest, village land, degraded wasteland and private land. Of the total area, 26% of forests are in the Aravali and Vindhyan formation ranges. The main forest types are of *Anogeissus pendula*, scrub and miscellaneous forests. In the peripheries, 432 sq. km. area is under agricultural crops and 55 sq. km. is lying fallow. As per the 1981 census, total population is 1,00,000 people living in 92 villages around the Ranthambore National Park. They are dependent on the park's resources mainly for small timber, firewood and charcoal requirements and grazing their cattle. Fodder is extracted from the buffer zone. Ranthambore is famous for its tigers and other predators: leopards, hyena, jackal, jungle cat and caracal. Deer and antelopes are sambar, spotted deer and chinkara and nilgai. Wild pigs are seen in advances have taken place in the field of molecular ecology as well as in the bioinformatics over the last several years. These advances have helped in understanding ecological, biological, socio-behavioral patterns in relation to genetic aspects of elusive and nocturnal species like carnivores and other wildlife species (Kohn *et al.*, 1999, Paetkau, 2003). Further developments in this field have also taken place in use of non-invasive based molecular scatology techniques in recent years which have helped in better understanding different ecological and biological aspects of the species which were not possible in the past. Therefore, with such development of non-invasive genetic sampling (NGS) in molecular ecology in combination with field data, it is possible for identifying species and individuals within population, population genetic diversity, survival rates, population density, sex ratio, home range, dispersal, distribution, paternity and kinship of free ranging animals (Kohn *et al.*, 1999, Taberlet *et al.*, 1996, 1999, Paetkau, 2003). Recently developed individual based Bayesian approaches have further helped in understanding population structure, gene flow and demographic history at landscape level (Chen *et al.*, 2007).

Habitat fragmentation has been major issue and when small animal population becomes fragmented and migration between subpopulation decreases or is limited, consequent increases in breeding and loss of genetic diversity can have serious negative impacts on the long term conservation. Therefore, studies related to population genetics are crucial for ensuring long term conservation of species especially in human dominated landscapes or in areas where resources are sparse (Dixon *et al.*, 2006, Manel *et al.*, 2007, Eckert *et al.*, 2008). Genetic technology offers a different approach from traditional field based methods in the study of social biology, providing otherwise unobtainable information about the behavioural interactions of cryptic, nocturnal or inaccessible species (e.g. Saarre *et al.* 2000). While genetic data can not reveal any information about social interactions that do not result in conception, these data can be a powerful approach for analyzing mating behaviour, through the ability to identify parents and their offspring. When combined with demographic data, genetic information has been used extensively to guide the management of many wild animals whether for the conservation of endangered species, or for the control of pests (e.g. Hogg and Forbs 1997; Gabor *et al.* 1999; Zenger *et al.* 2003). Understanding spatial structure is valuable if managers are to make informed decisions about how to control a population, such as which populations may be more important to target for control operations (Zenger *et al.* 2003, Hampton *et al.* 2004). Further most of this research has focused on rather small animals, such as insects or rodents ([illegible] 1976, 1978, Selander, 1976; Selander and Kaufman, 1973). Studies on large endangered mammals have been restricted to only few species (Baccus *et al.*, 1983 and Hartl, 1985).

C010

large numbers in the park and outside in agricultural areas and human settlements. Besides, it has variety of other fauna.

2.3 OBJECTIVES

This project has the following objectives:

1. To study genetic diversity in wild and domestic pigs in the vicinity of Ranthambore National park.
2. To find the level of hybridization between wild and domestic pigs based on genetic variability.
3. Based on the findings of the project, examine the possibility of hybridization between wild and domestic pigs in other parts of the country for further study.

2.4 JUSTIFICATION OF THE STUDY

1. The Project proposal is of high research priority of WII. The Project is the outcome of the research project 'Ecology and Management of wild pigs in and around Ranthambore Tiger Reserve.
2. The wild pig genetic resource is crucial for sustaining the domestic pig population in the country. So in case breeding and genetic hybridization between wild and domestic pigs is a serious issue, there would be an urgent need to examine genetic diversity of wild pigs in select protected areas and states in the country.
3. Wild pigs are straying out of Ranthambore national park and coming in contact with domestic pigs at the forest and village interface area. These domestic pigs have been found developing physical characteristics that resemble with wild pigs. Thus breeding and genetic hybridization between wild and domestic pigs is seriously impacting the wild population. Consequently, there is dissolution of wild genome and hybrids are produced.

   So there is an urgent need to study the genetic diversity in wild and domestic pigs in the vicinity of Ranthambore national park, and find the level of hybridization between them.
4. This management oriented study will provide a basis for developing mitigation strategy to address the problem of hybridization and help in long-term conservation of wild pig populations.

2.5 METHODS

Collection of tissue and blood samples

Blood samples will be collected from the jugular or ear vein of 30-40 individuals of core area and peripheral wild pig populations, and 15-20 physically restrained domestic pigs from the periphery of the national park. These blood samples will be collected in EDTA-containing vacuum tubes. These samples will be homogenized immediately and stored for processing further. In addition to this, tissue samples will also be collected using biopsy darts (Paxarms, New Zealand). These tissue samples will be stored and preserved following the techniques Waits *et al.* (2005). The tissue and blood samples will be collected from different areas viz. within national park, fringe areas and from outside where there is possibility of hybridization between wild pigs and domestic pigs. The selection of collection sites will be based on information gathered under the ongoing project on wild pigs and subsequent surveys to be done.

DNA extraction

Whole genomic DNA will be isolated by using QIAamp DNeasy Kit (QIAGEN) and Bio Robot for tissue and blood samples respectively as per suggested protocols by the manufacturer. Extracted genomic DNA from blood and tissue will be stored at -20^{0}C till further processing. Quality and quantity of DNA will be assessed by running samples on 2% agarose gel.

Appropriate molecular markers (mDNA and molecular DNA) will be used to examine the variation in mitochondrial DNA by sequencing the D-loop region in wild and domestic pigs and then discriminating hybrids, if any, among wild pig population in the vicinity of Ranthambore national park.

D loop amplification of mtDNA

Fragment of the D-loop region will be amplified by polymerase chain reaction (PCR) using the primers viz. MITL4 5' - CCAAAAACAAA GCAGAGTGTAC - and MITH4 5' – AGGCATTTTCAGTGCCTTG, as followed by Grossi *et al.* (2006). The PCR conditions will be followed as suggested by Grossi *et al.* (2006). The PCR reaction will be made in a final volume of 10 µl with the following: 3.0 mM $MgCl_2$, one unit of Taq DNA polymerase, 0.2 mM of each dNTP, PCR buffer 10X (200 mM Tris-HCl, pH 8.4, 500 mM KCl), and 5 pmol of each primer. Amplification cycles consisted of 34 cycles each, consisting of 94°C for 30 s, 60°C for 45 s and 72°C for 60 s. DNA fragment amplification will be confirmed by electrophoresis on 1% agarose gels stained with ethidium bromate and visualized under UV light. The purification of PCR products will be done by using commercial PCR purification kit of Qiagen, Inc., Germany. Then the fragment will be sequenced using the "Big Dye" kit (Applied Biosystems) on an automatic ABI 3130 DNA sequencer. All sequences will be analyzed using Bioedit software, version 7.0.0 and will also be edited based on the need.

Multilocus genotyping

Twenty three 23 FAO recommended molecular microsatellite markers (Behl *et al.*, 2002, 2006) will possibly be for multilocus genotyping data. Each marker will be synthesized and fluorescently labeled by ABI, Switzerland. For the microsatellite locus, PCR was set up in a 25 μl containing 1X PCR buffer, 2.5 mM $MgCl_2$, 250 μM of each of the four dNTPs, 0.4 μM of each of forward and reverse primers, 1 unit of Taq DNA polymerase (MBI Fermentas) and 50 ng/3 μl of the DNA extract. PCR Cycle conditions were as suggested by Behl *et al.* (2006) with initial denaturation at 94°C for 2 minute followed by 30 cycles of denaturation at 94°C for 45 s, annealing at optimum temperature (FAO 1998) for 45 s and extension at 72°C for 45 s with a final extension at 72°C for 10 minute.

All the PCR products were visualized on 2 percent agarose gel containing ethidium bromide and positive PCR samples will be run for genotyping on automated DNA sequencer model 3130.

Analysis of genetic variation and patterns of differentiation

The difference in nucleotide variation will be examined for samples collected from wild, domestic and hybrid pigs based on Chromatgram obtained from DNA sequencer as followed by Grossi *et al.* (2006). Based on comparison in variation in nucleotides, we will assess the feasibility of using variation in mtDNA differentiation in pigs for population differentiation as reported by Grossi *et al.* (2006).

The program Genetix 4.02 (Belkhir *et al.*, 2001) will be used to determine genetic variation by measuring allelic frequencies, observed (H_o) and expected heterzygosity (H_e). Deviations from Hardy-Weinberg (HWE) and linage (LE) equilibria will be evaluated using GENEPOP 3.2a (Raymond and Rousset, 1995).

The best suited software widely used for population differentiation including hybridization is "STRUCTURE" (Pritchard *et al.*, 2000). Pierpaoli *et al.* (2002) have also used this software to assess level of hybridization between wild and domestic cats. Therefore, multi-locus genotyping data obtained pigs will be subjected for analysis using program "STRUCTURE" to differentiate wild, domestic and hybrids if any in pig populations in and around the national park. In addition, we will also use Factorial Correspondence Analysis (FCA) which has been deployed for population differentiation and distinction (Pierpaoli *et al.*, 2002).

CP10

2.6 REFERENCES

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C810

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Zenger, K.R., Eldridge, M.D.B. and Cooper, D.W. (2003). Intraspecific variation, sex-biased dispersal and phylogeography of the eastern grey kangaroo *(Macropus giganteus)*. Heredity 91, 153-162.

2.7 WORK SCHEDULE (YEAR-WISE)

Activity	**Year I**	**Year II (3 months)**
Recruitment of Researcher and staff	—	
Capture of wild and domestic pigs and collection of blood samples.	———	
DNA extraction from blood samples and sequencing	———	
Data compilation and writing report		———

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STATEMENT-III

PROJECT BUDGET

Budget Head	Year I	Year II (6 months)
1. Fellowship and Wages		
1.1 Research Fellow (1) @ Rs. 12,000/- p.m. (JRF)	1,44,000	72,000
1.2 Field assistant (1) @ Rs. 2500/- p.m.	36,000	9,000
1.3 Casual labour	50,000	-
2. POL and maintenance of Vehicle		
2.1 Hiring of vehicles in field areas (Jeep/Gypsy/Tata Sumo)	4,50,000	50,000
3. Travelling Expenditure		
3.1 T.A. of Research team PIs, RFs etc.	1,00,000	25,000
4. Base camp expenses		
4.1 Accommodation in field area	50,000	10,000
4.2 Base camp establishment, electricity	25,000	-
4.3 Camping gears	50,000	-
5. Field/Lab. equipment		
5.1 Refrigerator/Defreeze	25,000	-
5.2 Miscellaneous items	25,000	-
6. Contingencies		
5.1 Immobilisation drugs, chemicals and glassware etc. for DNA extraction and sequencing	2,50,000	-
5.2 Darts, needles, accessories, medicaments	1,00,000	-
5.3 Medical expenses, lab. Invest. charges	50,000	-
5.4 Insurance	10,000	-
5.5 Advertisement	10,000	-
5.6 Report writing	20,000	-
5.7 Miscellaneous	50,000	-
6. Participation in workshop/symposium, conference etc.	50,000	
Total	**14,95,000**	**1,66,000**

Grand total: Rounded off to Rs. 16,60,000 *(Rupees Sixteen Lakh SixtyThousand only)*

JUSTIFICATION

The major part of the budget is for the enhanced fellowship, hiring vehicles for field work, immobilisation equipment, accessories and drugs and genetic markers and analysis costs. The petrol vehicle is essentially required to travel extensively in and around the park areas for identifying wild pig populations, monitoring their movement, capturing wild pigs and collecting blood samples. Darting syringes, needles and immobilization drugs are also expensive.

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(Proposal sent for reviewer's comments)

PROJECT TITLE

Study of genetic diversity in wild (*Sus scrofa cristatus*) and domestic (*Sus scrofa domestica*) pigs to find level of hybridization between them in the vicinity of Ranthambore National park.

1.	**Name & designation of the Investigators**	**:**	**Dr. N.P.S. Chauhan, Sr. Reader (Principal Investigator)** **Dr. S.P. Goyal (Co-Investigator)**
2.	**Address of Investigators**	**:**	**Wildlife Institute of India, P.O. Box 18, Chandrabani, Dehradun.**
3.	**Advisor**	**:**	**Director** **Wildlife Institute of India, P.O. Box 18, Chandrabani, Dehradun.**
4.	**Name of the Institution/ organization collaborating in the Project**	**:**	**Wildlife Institute of India** **Uttaranchal/Rajasthan Forest Departments**
5.	**Field locations**	**:**	**Ranthambore National Park.**
6.	**Dates & Duration of the Project**	**:**	**1 Year, 3 months (2009-10)**
7.	**WII's Research Development Plan: Thrust Areas**	**:**	**5.2 Monitoring of carnivores, prey populations and habitats** **5.5 Strengthening of wildlife forensic and conservation genetics capability**

8. Amount of funding requested:

Total amount of financial layout:	**Year I**	**Rs. 11,84,800.00**
	Year II	**Rs. 43,500.00**
	Total :	**Rs. 12,28,300.00**

Rounded off to Rs. 12,28,000.00 ***(Rupees Twelve Lakh Twenty Eight Thousand only)***

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9. Following documents are enclosed:

Statement I - Summary

Statement II - Project Proposal

2.1 Introduction
2.2 Study Area
2.3 Objectives
2.4 Justification
2.7 Methodology
2.8 References
2.7 Work schedule

Statement III - Project budget and justification.

Signature of Investigators

(N.P.S. Chauhan)

(S.P. Goyal)

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Study of genetic diversity in wild (*Sus scrofa cristatus*) and domestic (*Sus scrofa domestica*) pigs to find level of hybridization between them in the vicinity of Ranthambore National park.

Key words: Wild pigs, domestic pigs, breeding, genetic diversity, hybrids, conservation.

STATEMENT-I

SUMMARY

The Project aims at studying genetic diversity in wild (*Sus scrofa cristatus*) and domestic (*Sus scrofa domestica*) pigs to find level of hybridization between them in the vicinity of Ranthambore National park. From the year 2004 onwards, systematic study has been conducted on the ecology and behaviour of wild pigs in and around the park. It has been observed that wild pigs stray out of Ranthambore National park, raid agricultural crops and utilises the agro-ecosystem in peripheral villages for food resource and shelter. While this study was conducted, it was found that wild pigs were increasingly straying out of park into the forest-village interface areas and coming in contact with domestic pigs and perhaps breeding with them. As a result, there may be genetic hybridization between the wild and domestic pig populations. Domestic pigs normally have $2n = 38$ chromosomes and the wild pig has $2n = 36$. These domestic pigs have been found developing physical characteristics that resemble with wild pigs. Thus breeding and genetic hybridization between wild and domestic pigs is seriously impacting the wild population. Therefore it is very important to conduct a study on genetic diversity in wild and domestic pigs to find level of hybridization between them in the vicinity of Ranthambore National park. The project study on genetic diversity in wild and domestic pigs will provide a basis for long-term conservation of wild pig populations.

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STATEMENT-II

2.1 INTRODUCTION

The wild pig (*Sus scrofa scrofa*) is one of the most widely distributed large mammals. It has always been associated itself with man, and successfully utilises the human altered landscape (Fadeev, 1981 and Erkinaro *et al.*, 1982). Being a resilient and fast breeding animal, it is capable of expanding and establishing its population in new areas (Erkinaro *et al.*, 1982 and Ahmed, 1991). The species is also known for its biological and ecological plasticity (Tisdell, 1982 and Ramdas, 1987). From the year 2004 onwards, systematic study has been conducted on the ecology and behaviour of wild pigs in man-altered protected areas-village interface situations. Under the ongoing project, study on spatial and temporal distribution, diurnal activity, population status, socio-biology, habitat use, ranging patterns, seasonal changes in the dietary intake and feeding habits, nature and extent of agricultural crop damage, socio-economic aspects, reproductive biology and health aspects of wild pigs has been conducted. A wild pig-proof power fence and mass capture techniques for this species have also been developed.

Interestingly, it has been observed that wild pigs stray out of Ranthambore National park, raid agricultural crops and utilises the agro-ecosystem in peripheral villages for food resource and shelter. They have been increasingly coming in contact with domestic pigs (*Sus scrofa domestica*) in the forest-village interface areas and perhaps breeding with domestic pigs. As a result, there may be genetic hybridization among the wild and domestic pig populations. Domestic pigs normally have 2n = 38 chromosomes and the wild pig has 2n = 36 (Bosma, 1976). Despite these differences in chromosome number, these animals can mate and produce fertile hybrids. The hybrid has 37 chromosomes, and it is morphologically similar to the wild pig (Lui, 2000). Hybrids can successfully breed with the wild and domestic pigs or with other hybrids, generating offspring with 36, 37 or 38 chromosomes. Researchers have been studying and analyzing the genetic intraspecific and species-specific diversity of animals of economic interest and of wild animals. For studies of genetic differentiation, mitochondrial DNA (mtDNA) has been widely used as a tool to characterize races and determine genetic relationships (Brown *et al.*, 1989; Pegoraro *et al.*, 1996; Watanobe *et al.*, 1999). So it is very important to know the level of inbreeding between the wild and domestic pig populations in and around Ranthambore National park for conservation of this endangered species.

There is lot of emphasis to witness such phenomenon of genetic diversity and variability among variety of species. Increasing artificial alteration of landscape and intensive wildlife management may serve as potential forces for genetic change within populations of these species. Several advances have taken place in the field of molecular ecology as well as in the bioinformatics over the last several years. These advances have helped in understanding ecological, biological, socio-behavioral patterns in relation to genetic aspects of elusive and nocturnal species like carnivores and other wildlife species (Kohn *et al.*, 1999, Paetkau, 2003). Further developments in this field have also taken place in use of non-invasive based molecular scatology techniques in recent years which have helped in better understanding different ecological and biological aspects of the species which were not possible in the past. Therefore, with such development of non-invasive genetic sampling (NGS) in molecular ecology in combination with field data, it is possible for identifying species and individuals within population, population genetic diversity, survival rates, population density, sex ratio, home range, dispersal, distribution, paternity and kinship of free ranging animals (Kohn *et al.*, 1999, Taberlet *et al.*, 1996, 1999, Paetkau, 2003). Recently developed individual based Bayesian approaches have further helped in understanding population structure, gene flow and demographic history at landscape level (Chen *et al.*, 2007).

Habitat fragmentation has been major issue and when small animal population becomes fragmented and migration between subpopulation decreases or is limited, consequent increases in breeding and loss of genetic diversity can have serious negative impacts on the long term conservation. Therefore, studies related to population genetics are crucial for ensuring long term conservation of species especially in human dominated landscapes or in areas where resources are sparse (Dixon *et al.*, 2006, Manel *et al.*, 2007, Eckert *et al.*, 2008). Genetic technology offers a different approach from traditional field based methods in the study of social biology, providing otherwise unobtainable information about the behavioural interactions of cryptic, nocturnal or inaccessible species (e.g. Saarre *et al.* 2000). While genetic data can not reveal any information about social interactions that do not result in conception, these data can be a powerful approach for analyzing mating behaviour, through the ability to identify parents and their offspring. When combined with demographic data, genetic information has been used extensively to guide the management of many wild animals whether for the conservation of endangered species, or for the control of pests (e.g.

Hogg and Forbs 1997; Gabor et al. 1999; Zenger *et al.* 2003). Understanding spatial structure is valuable if managers are to make informed decisions about how to control a population, such as which populations may be more important to target for control operations (e.g., Zenger et al. 2003, Hampton et al. 2004). Further most of this research has focused on rather small animals, such as insects or rodents (Nevo, 1978; Selander, 1976; Selander and Kaufman, 1973). Studies on large endangered mammals have been restricted to only few species (Baccus *et al.*, 1983 and Hartl, 1985).

To get a first insight into the genetic structure and diversity of these straying wild pigs, investigations on domestic and wild populations are essentially required. Therefore we need to conduct a study on genetic diversity in wild (*Sus scrofa cristatus*) and domestic (*Sus scrofa domestica*) pigs to find level of hybridization between them in the vicinity of Ranthambore National park is necessary. The project study on genetic diversity of wild and domestic pigs will provide a basis for long-term conservation of wild pig populations. The results of this work will provide the database on genetics of wild and domestic pigs for use by wildlife managers and scientists dealing with such problems in different states.

2.2 STUDY AREA

The study will be conducted in and around the Ranthambore National park, which is situated in the Sawai Madhopur district of eastern Rajasthan. The total area of the Reserve is 1334.6 sq. km. and falls into the park area and its buffer, revenue land, reserve forest, village land, degraded wasteland and private land. Of the total area, 26% of forests are in the Aravali and Vindhyan formation ranges. The main forest types are of *Anogeissus pendula*, scrub and miscellaneous forests. In the peripheries, 432 sq. km. area is under agricultural crops and 55 sq. km. is lying fallow. As per the 1981 census, total population is 1,00,000 people living in 92 villages around the Ranthambore National Park. They are dependent on the park's resources mainly for small timber, firewood and charcoal requirements and grazing their cattle. Fodder is extracted from the buffer zone. Ranthambore is famous for its tigers and other predators: leopards, hyena, jackal, jungle cat and caracal. Deer and antelopes are sambar, spotted deer and chinkara and nilgai. Wild pigs are seen in large numbers in the park and outside in agricultural areas and human settlements. Besides, it has variety of other fauna.

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2.3 OBJECTIVES

This project has the following objectives:

1. To study genetic diversity in wild and domestic pigs in the vicinity of Ranthambore National park.

2. To find the level of hybridization between wild and domestic pigs based on genetic variability.

2.4 JUSTIFICATION OF THE STUDY

1. The Project proposal is of high research priority of WII. The Project is the outcome of the research project 'Ecology and Management of wild pigs in and around Ranthambore Tiger Reserve.

2. Wild pigs are straying out of Ranthambore national park and coming in contact with domestic pigs at the forest and village interface area. These domestic pigs have been found developing physical characteristics that resemble with wild pigs. Thus breeding and genetic hybridization between wild and domestic pigs is seriously impacting the wild population. Consequently, there is dissolution of wild genome and hybrids are produced.

   So there is an urgent need to study the genetic diversity in wild and domestic pigs in the vicinity of Ranthambore national park, and find the level of hybridization between them.

3. This management oriented study will provide a basis for developing mitigation strategy to address the problem of hybridization and help in long-term conservation of wild pig populations.

2.5 METHODS

Blood samples will be collected from the jugular or ear vein of from 30-40 individuals of peripheral and core area wild pig population's and 15-20 individuals of domestic pigs. These blood samples will be collected in EDTA-containing vacuum tubes. These samples were homogenized immediately. DNA was extracted and purified by applying the phenol-chloroform method (Sambrook *et al.*, 1989). Appropriate molecular markers (mDNA and molecular DNA) will be used to examine the variation in mitochondrial DNA by sequencing the D-loop region in wild and domestic pigs and then discriminating hybrids, if any, among wild pig population in the vicinity of Ranthambore national park.

Fragments of the D-loop region will be amplified by polymerase chain reaction (PCR) using the primers described by Okumura *et al.* (1996). DNA fragment amplification will be confirmed by electrophoresis on 1% agarose gels stained with ethidium bromate and visualized under UV light. The purification of PCR products was accomplished using the QiaexII Gel Extraction Kit (QIAGEN Inc.). The fragment will be sequenced using the "Big Dye" kit in an automatic ABI 377 sequencer (Molecular Dynamics).

2.6 REFERENCES

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2.7 WORK SCHEDULE (YEAR-WISE)

Activity	Year I	Year II (3 months)
Recruitment of Researcher and staff	–	
Capture of wild and domestic pigs and collection of blood samples.	——	
DNA extraction from blood samples and sequencing	———	
Data compilation and writing report		—

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